MICROCOPY RESOLUTION TEST CHART

LEVEL 1

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HISTOCOMPATIBILITY TYPING.

Annual rept.,

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HISTOCOMPATIBILITY TYPING

Background

In the course of performing relevant tasks Navy personnel
are frequently faced with potentially hazardous duty. These
hazards may take the form of physical trauma resulting in
severe limb injury or exposure to toxic materials or irradi-
ation, resulting in aplastic anemia. Physical trauma often
requires reparative surgery to reduce the morbidity associated
with the extensive injuries. Limb injuries, in particular,
are frequent among battle casualties. These wounds often
involve severe damage to nerves, vessels, bones, and muscles.
Even with modern methods of rapid evacuations, vascular surgery,
and surgical grafting techniques, full recovery of function is
often impossible. It has been the long-term goal of the Navy
to establish a process by which limbs might be transplanted
from cadaveric donors. For examples, fingers, hands, or feet
if transplanted in an appropriate environment and situation,
may result in the full functional recovery of the recipient.
In this way many injured individuals could be returned to full
activity or at least achieve much reduced disability. There
are a number of unsolved problems that currently preclude the
use of this therapeutic approach. The major unresolved barrier
to limb transplantation involves the immunological rejection of
the tissues that have foreign antigens. This is a problem

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common to all tissue and organ transplantation efforts and
represents the current state-of-the-art barrier to successful
transplantation of many types of limbs and organs. Part of
the solution to the problems of immunological graft rejection
lies in the area of improved tissue matching.

Military personnel are also at far greater risk of contact
with bone marrow toxic agents than the civilian population, by
virtue of working in hazardous areas. The necessity of
using various toxic fuels and chemicals as well as potential
exposure to irradiation, produce a hazard which requires the
availability of bone marrow transplantation in the treatment
of aplastic anemia from whatever source. Bone marrow trans-
plantation in cases of aplastic anemia has become an accepted
form of treatment and is currently funded in civilian insti-
tutions by health insurance plans. However, at this time,
the only long term survivors with this form of this therapy
have occurred when HLA identical siblings have been used as
the marrow donor. As only one third o f the potential trans-
plant recipients have matched sibling donors it is necessary
to identify methods of tissue matching which will allow the
use of unrelated donors. Improved methods of tissue typing
would then allow a much broader use of bone marrow transplan-
tation.

It is clear that the success of transplants, either organ or
bone marrow, is greatest with the most compatible grafts,
and is much less successful with poorly matched tissues. We

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have therefore initiated a program of tissue typing, in order to define the essential components of graft rejection and to seek ways to alter the body's natural rejection mechanisms.

Approximately one year ago we received a contract to study the human histocompatibility system and to develop methods for large-scale, accurate tissue typing for support of ongoing kidney and bone marrow transplantation programs within the Navy.

At the present time tissue typing is essential for all current transplant programs. However, it is clear that typing of histocompatibility antigens must be extended and further refined to fulfill its potential as a major clinical tool. To outline the complexity of the HLA tissue typing system, it now appears that there are at least four gene loci which control glycoprotein antigens on the human cell surface. These glyco-protein antigens are responsible for the rejection or acceptance of tissue grafts. These four loci have been called HLA-A, -B, -C and -D, and code for approximately 64 currently identified unique antigens. One of these, the HLA-D locus or LD (lymphocyte defined) locus, is the least understood; however, it seems to play a dominant role in the acceptance or rejection of the tissue graft. The research that we have initiated is directed toward the problem of examining this HLA-D system, defining the HLA-D locus antigens, and cataloging them in such a manner that individuals could be easily typed for these most important

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determinants. It is estimated that the seven currently accepted
HLA-D antigens represent about one fourth of the total, the
remainder have not been identified as yet.

Research Design and Plans

The initial plans for this contract were to follow a sequence
of events.

1. Establishment of a contract facility with a laboratory
   capacity to study the human histocompatibility system
   and do routine mixed lymphocyte culture testing.

2. Preparation of a panel of homozygous typing cells
   (HTCs) capable of identifying the common HLA-D
   specificities.

3. Preparation of panels of primed lymphocyte typing
   (PLT) cells for use in confirming the results of
   initial HTC typing, and for use when rapid knowledge
   concerning an individual's HLA-D type is essential.

4. Identification of alloantisera that will be capable
   of identifying the associated HLA-DR types in the
   standard microcytotoxicity assay.

5. Cryopreserving and storing these reagents in such
   quantities that they would allow typing of large
   numbers of individuals whenever necessary.

During the initial part of the contract our major goals
were to establish a functioning laboratory. The majority of the
necessary equipment is available, operative, and the laboratory
is functioning at an acceptable level. Certain essential equip-
ment, however, ordered during this fiscal year has yet to arrive

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and be installed. It is anticipated that these pieces of equipment and a tissue culture facility will be available for our use in the next three months. During the first year of this contract we recruited highly trained personnel and have established a tissue typing laboratory that is capable of performing high-quality HLA-D tissue typing. Our laboratory has become recognized internationally for its ability to accomplish this level of typing.

Data and Results

Accomplishments:

A. Establishing Reproducible Routine Typing Capacity

During the first year of this contract, a supply system for the procurement of human peripheral blood lymphocytes was established and routine mixed lymphocyte cultures were performed on a large number of unrelated individuals. This, then represented the groundwork for establishing a functioning HLA-D tissue typing facility.

B. Development of Methods for the Identification and Procure-
    ment of Homozygous Typing Cells (HTC)

1. The following is an overall approach to the identification and procurement of homozygous typing cells for this contract.

1.1 Identification of individuals with increased probability of HLA-D homozygosity.

1.1.1   Individuals who have been HLA-A and -B typed for other purposes and found to possess a single A and single B specificity are tested for HLA-D homozygosity. Table 1 depicts the current bank of cells from individuals with a single HLA-A

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and -B type that have been collected and stored frozen for testing. These individuals have an increased probability of HLA-D homozygosity for several reasons. First, there is a linkage disequilibrium between HLA-B and -D. Linkage disequilibrium is the increase in frequency of one allele (as identified by a specificity) of one genetic locus in the presence of a particular allele of a second linked locus. For example, while one would expect about 10% of HLA-B8 loci to be associated with HLA-Dw3, in actual fact about 60% association is found. Thus, according to the Hardy-Wienberg Law, which is a statistical method for determining gene frequencies, one would expect $(.16)^2$ or 36% of HLA-B8 homozygous individuals to be HLA-Dw homozygous. In practical terms, about 50% of HLA-A1, -B8 "homozygous" individuals have been found to act as typing cells for the Dw3 specificity. Likewise, about 25% of HLA-A3, B7 individuals act as typing cells for the Dw2 specificity. However, as one would expect, individuals homozygous for HLA-B specificities with lower HLA-B, -D disequilibria, are less likely to be HLA-D homozygous. Second, since individuals who have both a single A and B specificity are more likely to have identical HLA haplotypes by descent than individuals who have two different HLA-A loci and a single HLA-B specificity, one would expect a higher frequency of HTC donors among the HLA-A and -B homozygotes.

There are several sources of such HLA-A and -B homozygous individuals. Members of the serologic typing panels from local HLA-A and -B serotyping groups can be used. In addition, potential

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platelet donors for the Washington Area Red Cross and the National Institutes of Health are routinely HLA serotyped. We are collecting and cryopreserving lymphocytes from those platelet donors who are HLA-A and -B homozygous. These HLA panel members and platelet donors are an especially good source of HTC donors, as such individuals are generally willing to be leukophoresed, allowing the potential of collecting large numbers of typing cells. Using this method, 18 homozygous typing cell donors have been identified.

1.1.2   Inbred individuals, such as offspring of first-cousin marriages or members of highly inbred populations, have a greatly increased probability of HLA homozygosity. A group of Amish in Indiana and a sequestered population in North Carolina are currently being studied in cooperation with the Duke University Immunogenetics group. To date, two HLA-D homozygotes from the North Carolina group and two from the Indiana group have been identified. In addition to these two populations, nine HLA-D homozygous individuals have been identified among the Pennsylvania Amish in conjunction with the Johns Hopkins Immunogenetics group.

1.1.3   Families of kidney and bone marrow transplant patients and families studied for associations between HLA and disease who have been HLA serotyped are available from Duke University, the combined Army-Navy Transplant Service, and within our laboratories at Georgetown University School of Medicine. In cases where families are available, intrafamilial mixed leukocyte culture are performed. Unidirectional stimulation

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between parent and child is used as probable evidence for HLA-D homozygosity of the stimulating individual. Five HLA-D homozygotes have been identified using this technique.

1.1.4   Additional potential donors are individuals who have been typed for HLA-D, HLA-DR ("B" cell serotyping) and PL (primed LD typing) and found to have a single D, DR and PL specificity. Although these individuals may be HLA-A, -B and -C heterozygous, they have an increased probability of HLA-D homozygosity and, in some cases, lymphocytes from such donors act as HLA-D typing cells.

1.1.5   The use of the criteria and methodology set forth above has yielded 27 HTC of a wide variety of specificities (see HTC list, Table 2.)

1.2   Testing for functional HLA-D homozygosity.

1.2.1   Cryopreserved preparations are obtained from each donor. (See below for method of cryopreservation.) These cryopreserved samples are tested for functional ability to act as HLA-D typing cells. Samples are irradiated and used as stimulator cells in mixed leukocyte culture (MLC). These MLC are performed as relatively large matrices consisting of 10-60 stimulator cells (known HTC, possible HTC and control cells) and 10-60 responder cells (previously HLA-D typed cells selected for specificity, family members and previously undefined cells). The composition of the matrix varies enormously depending on the specificities being defined. For example, to initially identify the HLA-A1, -B8 homozygous cells for functional specificity as

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HLA-D3 typing cells, a matrix of ten stimulating cells (e.g., two known Dw3 HTC, five HLA-A1, -B8, -D? and three cells known not to possess HLA-D3) were tested against ten responder cells (e.g., five cells known to possess HLA-Dw3 and five cells known not to possess Dw3). The HLA-A1, -B8 cells, which type a majority of the responder cells, are then included in additional panels as stimulator cells to determine their reactivity against at least 30 responder cells. However, an HLA-A, -B homozygous cell with a lower linkage disequilibrium than those possessing HLA-B8 must be tested against a larger panel. For example, an HLA-A2, -B12 would need to bested along with a range of HTC, including HLA-Dw1 through Dw11, against at least 30 selected responder cells known to represent all defined HLA-D specificities and a number of undefined types. If the A2, B12 cell acts as a typing cell for only one common specificity (e.g., Dw1), further panel testing may be unnecessary. However, if the cell types a new specificity or produces nondefinitive results, it is retested on additional panels of responder cells For example, if a possible HTC typed two of three responder cells known to be Dw6, it would be repeated on another responder panel possessing these three Dw6 cells plus additional Dw6 cells and 10-30 additional non-Dw6 cells. However, because of the apparent crossreactivity and association among Dw2, 3, 5, 6, and 109, one might expect some ambiguity of typing for the Dw6 specificity. Where ambiguity of specificity of HTC exists, it can usually be cleared by (1) B-cell typing, (2) use of these cells as responder cells in HTC experiments, (3) reciprocal testing against a panel of typing cells of that particular specificity, and (4) primed LD typing or PLT test (primed lymphocyte typing test).

In all cases where family members are available, the proposed HTC donor is tested in reciprocal MLR against their parents or children and siblings. Nonstimulation of the appropriate relative by proposed HTC donor is strong evidence for HLA-D homozygosity. However, frequently family members are not available. Using the PLT test, this laboratory has identified HTC for Dwl through Dw4, from individuals where family members were not available, which far exceeds the consistency and relative discriminating ability of cells available in the Seventh International Histocompatibility Workshop.

1.2.2   Primed LD typing is performed where necessary, to assure homozygosity and appropriate assignment of specificity. The proposed HTC is used as a primary phase stimulator cell against a primary phase responder cell which differs for the HLA-D specificity (e.g., Dwl HTC responder against proposed Dw4 HTC as stimulator). This primed combination would then be tested in a secondary phase against a panel containing cells of all known HLA-D specificities and a number of undefined loci. Generally, each specificity is represented by two to five members of the secondary stimulator group. Restimulation of the primed cell combination with the cells which do not possess the appropriate -D specificity is evidence against homozygosity of the proposed HTC. Restimulation of the appropriate primary phase combination with cells of the correct HLA-D specificity is collaborating evidence of correct HLA-D specificity assignment.

1.3   Collection and cryopreservation of proposed and defined HTC. (See Table 3 for Flow Chart.)

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1.3.1   The collection of cells by venipuncture from
normal, healthy volunteers is carried out
using the rules and guidelines as set forth by the American
Association of Blood Banks (AABB) Technical Manual. Sedimented
leukocytes from plateletpheresis procedures from all donors
with only two antigens are collected daily. Other prospective
HTC donors from the Washington, D.C., area come to the George-
town laboratory, or alternatively the Georgetown technician
obtains the sample on site in the donor's home or nearby medi-
cal facility. For initial testing, a 50-100 cc sample is ob-
tained. Members of the heterozygous HLA-D typing panel whose
cells have been well characterized each donate blood in 500-ml
units. Once an acceptable HTC donor is identified and speci-
ficity control performed, they are leukophoresed using either
the Fenwall "Quadpack" technique or the Haemenetics Model 30
cell processor.

Donors from remote areas donate blood or are leukophoresed
at nearby towns by arrangement with local hospitals or blood
banks, or are transported to Washington, D.C., for leukophor-
esis. Simple donation of blood can generally be handled
locally and the blood shipped to Washington by Air Carrier or
Express delivery. However, in cases where the donor lives in
a remote area, a Georgetown technician may need to travel to
the local area to obtain blood. When leukophoresis is per-
formed in a remote location, it is usually necessary for a
technician or a physician to travel to the leukophoresis site
to supervise the collection and hand carry the sample for
cryopreservation in Washington.

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1.3.2   Cells are cryopreserved by obtaining lymphocytes relatively free of red blood cell, granulocyte and platelet contamination by the Ficoll-Hypaque centrifugation gradient technique, followed by differential centrifugation of the sample. Lymphocytes thus obtained are cryopreserved in a medium consisting of RPMI with 25 mM HEPES, Gentamycin, 10% human AB plasma (or fetal calf serum) 7.5% DMSO (dimethyl sulfoxide) and 10 units per ml heparin. Cells are frozen in 0.4-ml aliquots in 1 ml Wheaton type A boro-silicate glass vials with aluminum crimp tops and injectable Teflon liner at a concentration of 6-15 x 10^6 per vial.

1.4   Statistical analysis. Data obtained is transmitted to the NIH computer facility and stored on disc and tape. Although no statistically proven method of analyzing HLA-D data has been accepted, one ad hoc technique, "double normalization," using an upper 75th percentile for both responder and stimulator corrections has been devised. In reality, this method serves a very useful purpose of allowing simplified visual clustering into responder and nonresponder areas. The data from this laboratory is currently analyzed using a computer program written in the language PL-1 for the IBM 360/370 computer. Data is expressed as median values and double-normalized values (DNV) of these medians. DVN less than 40 are considered typing responses and values greater than 40 as nontyping responses. Because of variability of typing responses most lymphocyees must be typed with two to six HTC for each known specificity to assure a high level of reliability.

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C.   Establishment of Cryopreservation Techniques for
      Long Term Storage of HTC

Unique methods of obtaining large numbers of viable lymphoid
cells and freezing them in aliquots have been developed. It was
found that up to 6×10^9 viable lymphocytes could be obtained
from a single donor using the Hemonetics Model 30 cell processor.
To collect these large amounts of lymphoid cells, the machine is
set up as it is normally used to collect platelets. A venipuncture
is performed and the centrifugation-collection bowl filled at
30-70 ml per minute. Collection of lymphoid cells is started by
diverting the outflow from the bowl as soon as the outflow line   •
becomes pink from the first appearance of red blood cells. The
lymphoid cell collection is continued for two minutes at an outflow
rate of 20 ml per minute. At the end of that two minute collection,
the centriguation-collection bowl is stopped, the plasma and red
blood cells returned to a reinfusion bag and returned to the donor
by gravity flow. After the first lymphocyte poor unit of blood is
returned, up to six additional lymphocyte collection cycles are
performed.

Up to 350 cc of lymphocytes in donor plasma and trisodium-
citrate anticoagulant must then be processed to remove unwanted
granulocytes, plates, and red blood cells. To perform this, the
lymphocyte rich preparation is diluted 2:1 with RPMI 1640 with
25 mM HEPES and 30 units per ml Heparin. The diluted preparation
is layered on Ficoll-Hypaque (specific gravity 1.078) and centri-
fuged at $22^{\circ}C$ for 40 minutes at 400 x G. The lymphocytes and

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platelets, now free of red blood cells and granulocytes, are removed from the interface, transferred to 50 ml centrifuge tubes and diluted 1:1 with tissue culture medium and centrifuged for 15 minutes at 250 x G. This low speed centrifugation removes up to 90% of the platelets.

The lymphocytes are counted and diluted to a concentration of 30 x 10^6 per ml with tissue culture medium containing 10% AB plasma and 10 units per ml of heparin. The cell suspension is cooled to 4°C and an equal volume of cold tissue culture medium with 10% AB plasma and 15% dimethyl sulfoxide is then added. The cells are aliquoted in 0.4 ml volumes into 1 ml type A boro-silicate glass vials. The top of these vials is closed with aluminum crimp tops with teflon lined injection ports. The cells are then frozen in a controlled rate freezer at -1°C per minute to -30°C then -5°C per minute to -100°C and transferred to a liquid nitrogen freezer. Cells obtained in this manner and main-tained at -130° to -196°C should be functional for up to 10 years.

We have established a method for irradiating lymphocytes while the cells are maintained at -80°C in dry ice. We found that twice the ambient temperature radiation dose is required to produce the identical effect upon lymphocytes maintained in dry ice. This is done to the low temperature, reducing molec-ular movement and thus reducing the ability of free radicals generated by irradiation to damage chromosomes by cross linking of DNA. This method is of considerable importance in HTC typing,

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as it allows a significant saving of time during the critical
phases of performing large matrix experiments.

D.   Development of the Hybrid HTC-PLT System, for the Rapid
      Identification of HLA-D Types

Because of our capacity to obtain large numbers of
homozygous typing cells (HTC), we were able to use these
HTC to develop a hybrid system of primed lymphocyte testing
(PLT). The PLT is a system whereby lymphoid cells from one
individual (primary responder) can be primed to lymphoid
cells from a second individual (reference donor). Memory
cells are generated in vitro to the lymphocyte defined (LD)
antigens of the reference cell donor. By using these genetic-
ally restricted HTC in the PLT, primed cells which were
extremely selective for the appropriate antigens could be
generated.

PLT testing offers a number of potential advantage over
HTC testing. First, results of the testing can be available
within 48 hours of performance of the test (and potentially
within 24 hours). Second, smaller numbers of cells can be
used. Third, new and rare LD types can be defined much more
quickly using PLT than HTC method. The PLT cells can be stored
in the frozen state indefinitely.

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E.   Current Status of Serotyping for DR Specificities

At this time, serotyping for HLA-DR is performed in conjunction with the Duke University Division of Immunology. Under an informal cooperative agreement, this laboratory has performed HLA-D cellular typing for the Duke University group and this group has performed serotyping for the Georgetown contract at no cost. This cooperative approach has allowed the rapid advancement of HTC and PLT typing under this contract. We will continue to place our major emphasis on HTC and PLT typing until the majority of specificities defined by these methods have been identified and we are able to maintain adequate numbers of typing cells. Once this is accomplished, it will be relatively simple to obtain HLA-DR antisera through cooperative efforts with other laboratories and simplified screening procedures afforded by our possession of HLA-D and -DR defined lymphocytes.

F.   Refinement of HLA-D Region Immunogenetics

This laboratory has developed extensive capabilities in the cell-typing area. We have been major participants in the Seventh International Histocompatibility Testing Conference held in Oxford, England, and are part of the U.S. Region I Histocompatibility Testing Group, which performed numerous cooperative functions for the International Workshop. The regional organization has continued after the International

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Workshop and our laboratory continues in its position as the major contributor for HTC and PLT typing. In addition to gaining expertise and well characterized, typed, random cell panel, during the Seventh Workshop, we have exchanged cells with a number of other laboraotries, allowing us to routinely type for all currently accepted specificities. We are the American reference laboratory for HLA-Dw2.

In the process of developing an HLA-D typing facility, our group has played an important role in expanding knowledge concerning the HLA-D genetic region. As major participants in the Seventh International Histocompatibility Workshop, we contributed to the identification of three previously undefined HLA-D specificities. Our laboratory was particularly important in demonstrating that the new specificity Dw11 is a subset of the established HLA-Dw7 specificity. We have demonstrated the cross reactivity of Dw5 with Dw6 as well as Dw1 with Dw8. In addition we have shown a linkage disequilibrium between the Dw2 and Dw6 specificities and between Dw3 and Dw7.

In collaborative studies with the Duke University Immuno-genetics group we have established a relationship between three different methods of HLA-D region typing and the most likely genetic basis for these relationships. First, our laboratory and several other laboratories showed that the HLA-D determinants (as defined by HTC typing) and the HLA-DR determinants (as defined by B cell serology) traveled together on the same

major histocompatibility locus haplotype. Second, the HLA-D
and HLA-DR specificities were shown to be associated in approx-
imately 90% of haplotypes tested. Individuals and families who
did not type for the same HLA-D and -DR specificities were care-
fully studied by performing numerous repeat HTC testings and
B cell serologic testings, as well as serum adsorption studies.
Furthermore, primed LD typing was performed on certain individuals
and families who did not type for both the HLA-D and associated
HLA-DR specificity. The repeated HTC and B-cell serology testing
confirmed the appropriate assignment of specificity. Serum
adsorption reactions. By far, the most likely explanation for
this dichotomy of HLA-D and HLA-DR typing is that these represent
two distinct specificities encoded by two separate but closely
linked genes on the sixth chromosome.

Additional corroborating evidence for the dichotomy of the
HLA-D and -DR specificities was provided by this laboratory using
the primed LD typing test. Using a family with established dif-
ferences between HLA-D and -DR haplotype assignments, it was
shown that lymphocytes could be primed in vitro to the HLA-DR
specificity independently of the HLA-D specificity and conversely
could be primed to the HLA-D specificity exclusive of HLA-DR
specificity. These studies are depicted in Table 4. This finding
is of enormous significance for several reasons, in addition to
the confirmation of HLA-D, -DR dichotomy. First, it demonstrates
a greatly expanded potential for PLT typing by demonstrating its
ability to define genetic subregions to a degree previously felt
not possible. This will lead to the use of the PLT in defining

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suspected genetic subregions in other parts of the major histo-compatibility complex (MHC) as well as possible non-MHC genetic subregions. Second, it is the first demonstration of the ability of a cell surface antigen which is both capable of stimulating antibodies (HLA-DR antisera) to allogeneic specificities and also inducing memory cells in vitro. It should be noted, however that this second point is not completely established at this time, as the postulated -DR specificity identified in PLT could be generated by a third -D region genetic locus which was more closely linked to -DR than -D. However this ability of the PLT to detect very small antigenic differences may lead to methods of predicting allograft rejection prior to clinical transplantation, detecting early phase rejection crises and identifying graft versus host disease. As results from PLT experiments can be available in less than 48 hours (and potentially less than 24 hours) these findings may lead to clinically useful tests. Further, because of its discriminatory ability, PLT may lead to the detection of currently defined genetic regions important in transplantation, transfusion and disease prevention.

G.   Typing of Random Donors

Utilizing the techniques that we have developed, including the HTC panel, and the PLT test, it is now possible to type about 30% of the HLA-D genes and type both alleles in any one

donor in 10-15% of the population. It is estimated that over the next two or three years the number of random individuals in whom both alleles can be successfully typed will increase substantially, as the availability and variety of homozygous typing cells expands.

H.   Publications and Presentations

1.   On the basis of our initial success and of the interest of the Naval Medical Research Institute in this project, our group, in collaboration with NMRI investigators, was able to organize an international meeting entitled "The First International Workshop on Human Primed LD Typing" (Bethesda, Maryland, January, 1977). A summary of the meeting is included. It is anticipated that this will be published in Transplantation Proceedings.

2.   Investigators receiving support from this contract were invited participants in the 7th International Histocompatibility Workshop (Oxford, England, September, 1978) where two abstracts, one poster and a formal paper were presented (attached).

3.   In addition, investigators supported by this contract were also invited participants in the "Annual Southeast Organ Preservation Society Meeting," May, 1978, where a presentation entitled "HLA-D Typing by MLC and PLT" was made.

Proposal for Continuation of this Contract

The initial success of this contract has provided evidence
that continued investigation would soon allow us to enlarge the
number of random individuals who can successfully be typed. It
is estimated that the HLA-D loci code for 25-30 antigens, 11 of
which have been identified. It is proposed that work continue
along the lines of identifying the new specificities, securing
a source of HTCs, and generating the PLTs so that a high number
of individuals could be adequately typed. As sufficient new
cells are available, frozen cell panels will be established and
stored for subsequent use.

We would propose to continue the work scope essentially as
outlined in the Research Design and Plans (page 6), identifying
cells with new specificities, storing them, and including them
in our HTC and PLT panels for increased typing capacity. These
cells will be made available to the tissue typing program of
the Naval Medical Research Institute, as well as to other ONR
contracts dealing with the development or expanded use of HLA
type cells for transplantation or other areas of interest.

Parts 1, 2, 3, and 5 of our Research Designs and Plans
(page 6) are well under way and it is anticipated that part 4,
the screening of HLA-DR alloantisera, will begin on a limited
scale during the latter part of the next fiscal year. It is
estimated that this project will require approximately five
years of work to establish the panels necessary to type the
majority of random individuals.

Table 1

Summary of Inventories of Frozen Cells Categorized by Serological HLA Types

Cells Homozygous at HLA-A and -B loci		No. Collected	Identification No.
HLA-A	HLA-B		
1	8	35	139, 110, 120, 106, 158, 17, 03, 99, 69, 147, 10, 144, 09, 146, 13, 70, 05, 16, 180, 129, 90, 213, 214, 29, 28, 06, 215, 226, 230, 234, 235, 240, 249, 250
2	12	23	161, 212, 183, 221, 190, 206, 14, 143, 111, 198, 53, 133, 94, 97, 91, 86, 105, 22, 238, 245, 257, 290, 204
3	7	16	76, 75, 07, 116, 134, 135, 130, 08, 119, 02, 04, 216, 117, 222, 239, 277
2	7	6	01, 140, 121, 92, 85, 231
29	12	7	09, 155, 104, 40, 27, 253, 74
2	40	6	151, 157, 154, 172, 149, 64
2	27	3	162, 153, 150
2	5	4	159, 14, 36, 24
11	35	2	148, 30
2	17	3	42, 43, 41
2	15	5	177, 15, 227, 229, 232
2	8	3	93, 24, 218
32	8	2	89
1	17	1	145
28	35	1	228
24	35	1	152
3	14	2	56, 118
9	12	1	201
1	35	2	195
3	35	2	223, 254
30	13	1	237
2	18	2	256
1	12	1	272
24	12	1	315
24	7	1	221

Table 2

Current HTC Typing Panel for HLA-D Antigens

	Identification No.	Laboratory of Origin	HLA A	HLA B	HLA D
For Dw1	14	Local	2	5	1
	30	Local	11	35	1
	172	Local	2	40	1
For Dw2	02	Local	3	7	2
	01	Local	3	7	2
	140	Local	2	7	2
For Dw3	03	Local	1	8	3
	29	Local	1	8	3
	250	Duke	1	8	3
	10	Local	1	8	3
For Dw4	91	Local	2	12	4
	22	Duke	2	12	4
For Dw5	94	Local	2	12	5
	TERH	Leiden			5
	7W569	Tubingen			5
	7W554	Milan			5
For Dw6	HAAK	Leiden			6
	JG	Dallas			6
For Dw7	7W534	Johns Hopkins	1,2	14	7
	7W542	Minnesota			7
For Dw8	7W540	Venezuela			8
	ERIK	Oslo			8
	FERR	Milan			8
For Dw9	118	Local	3,33	14	9
For Dw10	7W543	Boston			10
	7W548	Sloan Kettering			10
For Dw11	7W535	Johns Hopkins	2,9	17	11
	7W533	Johns Hopkins	29	12	11
	40	Local	29	12	11

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Table 3

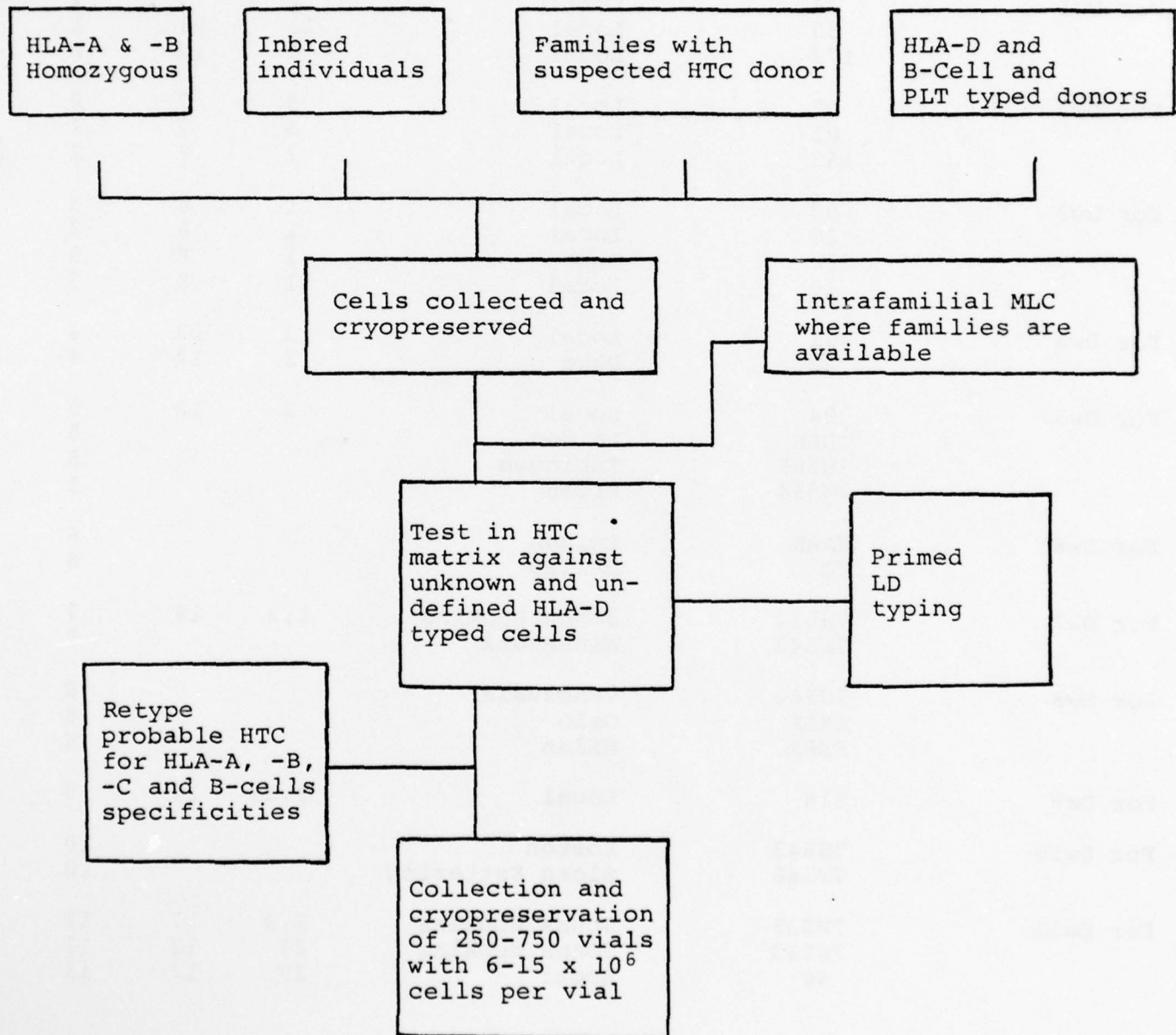Schematic Diagram of Proposal for

Identification and Procurement of HTC

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┌──────────────┐  ┌──────────────┐  ┌──────────────────┐  ┌──────────────────┐
│ HLA-A & -B   │  │ Inbred       │  │ Families with    │  │ HLA-D and        │
│ Homozygous   │  │ individuals  │  │ suspected HTC    │  │ B-Cell and       │
│              │  │              │  │ donor            │  │ PLT typed donors │
└──────────────┘  └──────────────┘  └──────────────────┘  └──────────────────┘
```

```
┌──────────────────────┐          ┌──────────────────────┐
│ Cells collected and  │          │ Intrafamilial MLC    │
│ cryopreserved        │          │ where families are   │
│                      │          │ available            │
└──────────────────────┘          └──────────────────────┘
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```
┌──────────────────────┐          ┌──────────────┐
│ Test in HTC          │          │ Primed       │
│ matrix against       │          │ LD           │
│ unknown and un-      │          │ typing       │
│ defined HLA-D        │          │              │
│ typed cells          │          │              │
└──────────────────────┘          └──────────────┘
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```
┌──────────────────────┐
│ Retype               │
│ probable HTC         │
│ for HLA-A, -B,       │
│ -C and B-cells       │
│ specificities        │
└──────────────────────┘
```

```
┌──────────────────────────┐
│ Collection and           │
│ cryopreservation         │
│ of 250-750 vials         │
│ with 6-15 x $10^6$       │
│ cells per vial           │
└──────────────────────────┘
```

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Table 4

Complete Major Histocompatibility Complex (MHC)

Typing of One Family

			Father					Mother	
	Haplotype		a	b				c	d
	HLA-A		2 $\overline{A}$	$\overline{A}$ 1			30 $\overline{A}$	$\overline{A}$ 26
	HLA-C		α $\overline{C}$	$\overline{C}$ β			γ $\overline{C}$	$\overline{C}$ δ
	HLA-B		5 $\overline{B}$	$\overline{B}$ 7			12 $\overline{B}$	$\overline{B}$ 38
PL_D	=	HLA-D	* $\overline{D}$	$\overline{D}$ 2			7 $\overline{D}$	$\overline{D}$ **
PL_{DR}	=	HLA-DR	2 $\overline{DR}$	$\overline{DR}$ 2			7 $\overline{DR}$	$\overline{DR}$ 4x7

Sib #1	Sib #2	Sib #3	Sib #4	Sib #5
2 $\overline{A}$ $\overline{A}$ 30	2 $\overline{A}$ $\overline{A}$ 30	2 $\overline{A}$ $\overline{A}$ 26	1 $\overline{A}$ $\overline{A}$ 26	2 $\overline{A}$ $\overline{A}$ 26
α $\overline{C}$ $\overline{C}$ γ	α $\overline{C}$ $\overline{C}$ γ	α $\overline{C}$ $\overline{C}$ δ	β $\overline{C}$ $\overline{C}$ β	α $\overline{C}$ $\overline{C}$ δ
5 $\overline{B}$ $\overline{B}$ 12	5 $\overline{B}$ $\overline{B}$ 12	5 $\overline{B}$ $\overline{B}$ 38	7 $\overline{B}$ $\overline{B}$ 38	5 $\overline{B}$ $\overline{B}$ 38
* $\overline{D}$ $\overline{D}$ 7	* $\overline{D}$ $\overline{D}$ **	* $\overline{D}$ $\overline{D}$ **	2 $\overline{D}$ $\overline{D}$ **	* $\overline{D}$ $\overline{D}$ **
2 $\overline{DR}$ $\overline{DR}$ 7	2 $\overline{DR}$ $\overline{DR}$ 4x7 §	2 $\overline{DR}$ $\overline{DR}$ 4x7	2 $\overline{DR}$ $\overline{DR}$ 4x7	2 $\overline{DR}$ $\overline{DR}$ 4x7

§ represents a genetic cross over

α β γ & δ are undefined HLA-C types

* and ** represent previously undefined HLA-D (as defined by HTC typing) and PL_D (as defined by primed LD typing) specificities. No HTC has yet been found to define these new types, however, specific PLT cells have been generated.

SUMMARY OF THE FIRST INTERNATIONAL
WORKSHOP ON HUMAN PRIMED LD TYPING

NAVAL MEDICAL RESEARCH INSTITUTE
BETHESDA, MARYLAND

Acknowledgements

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It is now recognized that the proliferative response in mixed leuko-
cyte cultures (MLC's) is due primarily to the recognition by responder
cells of lymphocyte defined (LD) antigens on the surface of stimulator
lymphocytes. The currently known LD antigens are encoded by a locus,
within the major histocompatibility complex (MHC) located on the 6th
chromosome, which has been termed the HLA-D locus as distinguished from the
serologically defined (SD) closely linked HLA-A, B & C loci. There has
been considerable interest in the ability to determine the genetic poly-
morphism of the HLA-D locus and nearby regions. Recently, a technique has
been described which provides a powerful tool for the examination of this
genetic polymorphism. The test is called the primed LD typing (or
primed lymphocyte typing) (PLT) test and is based on the observation
that lymphocytes can be primed with allogeneic lymphocytes in vitro
(Hayry & Anderson 1974, Zier & Bach 1975, Fradelizi & Dausset 1975).
When these primed cells are restimulated with the antigens to which they
were originally sensitized, a vigorous secondary response is observed.
It was determined that human lymphocytes primed in vitro to cells dif-
fering by a single HLA haplotype (or HLA-D region determinant) and
incubated for 9-14 days can be restimulated to respond in a secondary
manner by cells carrying the same HLA-D region antigens present on the
initial sensitizing cell. The unknown cells which give rise to such a
secondary response therefore possess similar if not identical LD antigens
to the priming (reference) cell. This procedure has led to a method for
typing of surface antigens encoded by the HLA-D region (Sheehy et al.
1975, Crosier et al. 1977, Hirschberg et al. 1975). This assay is
currently being used in various laboratories around the world. Members
of 17 laboratories participated in a conference on PLT typing held at
the Naval Medical Research Institute, Bethesda, Maryland. This is a
summary report from that workshop.

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I.   Technical Aspects

   (a) Methods

   Although numerous modifications of the basic method have been
successfully employed, all current methods basically incorporate the
technique initially described by Sheehy (Sheehy & Bach 1975).

   Primary Sensitization: Responding lymphocytes and mitomycin-
treated or X-irradiated stimulating cells are usually co-cultured in 20
ml of tissue culture medium in 50 ml tissue culture flasks (Falcon Model
3013) standing upright. However, primed cells have been produced using
as little as 6 ml media and 2×10^6 responder and stimulator cells in
tissue culture tubes, or as much as 200 ml of media with 400×10^6
responder and stimulator cells in 2 liter roller bottles.

   The primed cells are harvested on day 9-14; some laboratories add
or exchange media during incubation. Cell yields are usually equal to
the number of responder cells placed in culture, however may vary from
less than half to four times the number of initially cultured responders.

   Secondary Stimulation: Primed lymphocytes are dispensed into
V-bottom or round bottom 96 well microtiter tissue culture trays for
secondary stimulation. Generally $12.5-100 \times 10^3$ primed responding cells
(usually 25×10^3) are cultured with $12.5 - 200 \times 10^3$ mitomycin-C
treated, X-irradiated or untreated fresh stimulating lymphocytes (usually
50×10^3) in a total volume of 0.15-0.2 ml of tissue culture medium per
well. Secondary responses are assessed after incubation for 24-72 hrs
at 37^oC in a 5% CO_2 atmosphere, by pulsing with 1-2 μCi ^3H-TdR (2-60
Ci/mM) per well. Six to 18 hours after pulsing, the cells are harvested
on filter paper using a sample harvester (Hartzman et al. 1972,
Hirschberg et al. 1975) and the radioactive incorporation determined
with a scintillation counter.

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Tissue Culture Media. RPMI 1640 buffered with both bicarbonate and HEPES (25 mM) is used by most laboratories. The medium is usually supplemented with 5-20% pooled human plasma or serum. Antibiotics, usually penicillin (100 μ/ml) with streptomycin (100 μg/ml) or gentamycin alone (50 μg/ml) are also added to the media, as well as fresh L-glutamine.

INTERPRETATION OF RESULTS

Those populations of stimulating cells that induce a secondary response of similar magnitude to the primary stimulating (reference) cell are considered "positive". Those cells that induce proliferation comparable to that stimulated by the primary phase responding individual (control) are considered "negative". In reality, the great majority of stimulating cells induce secondary responses greater than the negative control but less than response to the reference cell. These intermediate responses produce much of the problem in analyzing PLT data. It was felt that intermediate level stimulation may reflect either of two phenomena. First, it could reflect a partial antigen sharing or cross-reactivity by determinants on the test stimulator with that of the reference cell. Second, these intermediate responses might represent "accelerated primary responses" to LD antigens not recognized on the initial stimulator, presumably mediated by responding lymphocytes that had survived in an inactivated state for the 9-14 days of primary sensitization. It was concluded that these two possibilities were not mutually exclusive and that no clear evidence refuting either theory had been yet produced.

Technical Modifications

Several technical modifications were suggested to enhance the "discriminatory" ability of secondary cultures. Hirschberg and Thorsby

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added 2-mercapto-ethanol to primary cultures. The cells produced by this technique gave relatively weak secondary responses, but produced a greater separation of apparent stimulatory ("positive") and non-stimulatory ("negative") secondary responses. Wank reported on the use of low numbers of primed cells (12.5×10^3) in the secondary phase culture. The use of a decreased number of responding cells appeared to lower the "non-specific" restimulation. Mawas, however, tried the same "titration" approach without influencing the discriminatory ability of these cells. Repeated priming in MLC has been attempted by several groups. Crossier found that one could markedly increase the cell yield by repeated stimulations with a single stimulating individual, and occasionally show an increased specificity of these multiply primed cells (Crosier et al. 1977). Numerous schedules for harvesting of the secondary phase cultures were assessed. Some groups reported greater discrimination by early (24 hr) secondary phase harvesting while others reported no consistent improvement in early versus late (48-72 hr) harvesting. Late harvesting offers some technical advantage in that generally higher CPM are obtained, reducing radioactive counting error; however, early harvesting may produce greater selectivity. The problem of identifying the optimum duration of the secondary phase culture is complicated by certain cell combinations which produce peak responses at 24 hrs while others do not peak until 72 hrs of culture. Reinsmoen has used multiple harvesting intervals to avoid the problem of optimal secondary culture duration. No consensus of opinion was obtained.

Several groups have attempted to select for those lymphocytes actively responding in the primary culture. The Paris group (Fradelizi et al. 1977) has separated the theoretically committed blasts from the small lymphocytes on 1G sedimentation gradients during maximum proliferation

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of the primary phase culture (5-7 days). The separated cells were
further cultured for several days and then harvested. The blast cell
derived population showed discriminant specificity upon secondary chal-
lenge, although the discriminatory potential was not improved. On the
other hand, the small lymphocyte isolated population from the primary
phase, when challenged in the secondary phase, showed no specificity.
These data indicate that the blast cells are the progenitors of the
cells that give rise to discriminatory PLT responses. Rimm and Bach,
using a similar unit gravity separation of blast cells, allowed the
blast cells to revert to small lymphocytes. They presented preliminary
data consistent with increased discrimination with the separated cells
as compared with nonseparated cells.

In efforts to determine similarities and/or differences between
the responder cells in primary MLR from those in the secondary MLR,
Hartzman, Pappas and Sell used a heterologous cytotoxic antisera against
human T cell specific antigen (HTLA). No MLC reactivity was seen when
responder cells were pretreated with this antisera and guinea pig
complement prior to primary culture. Interestingly, pretreatment of the
primed lymphocytes with an amount of antiserum which completely abolished
the primary MLR was neither cytotoxic to primed lymphocytes nor altered
the secondary proliferative response. Furthermore, treatment of PLT
cells with this antisera did not reduce the so called non-specific
activation of these cells. Lack of suppression of non-specific response
by anti-HTLA treated cells lends support to the theory that low and
middle level PLT re-activation is not due to a rapid primary MLR from
unprimed lymphocytes in the secondary phase of the PLT.

As a practical approach to generating large numbers of primed
cells, a cooperative study by Yunis and Bach was performed to look at

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priming with lymphoblastoid cell lines generated from homozygous typing cells (Reinsmoen et al. 1977). Such cell lines are a virtually limitless source of HLA-D homozygous cells, but have the associated difficulty of inducing autostimulation of normal lymphocytes from the cell line donor. In the primary phase PLT, unrelated responding cells were stimulated with homozygous lymphoblastoid cell lines, becoming primed to both HLA-D and the "autostimulating determinant". Secondary responses of these cells were tested with a panel of normal stimulating lymphocytes, thus testing only for restimulation by the histocompatibility antigens. Results from PLT cells primed initially with lymphoblastoid cells were highly correlated with results from cells primed with normal homozygous cells.

The issue of "autostimulatory" antigens on lymphoblastoid cell lines was studied in a tumor model with PLT by Reinsmoen. Some Leukemia cells (both ALL and AML) were shown to stimulate weak proliferative responses of either autologous remission lymphocytes or lymphocytes from MHC identical siblings. Restimulation of these cultures with autologous and allogeneic leukemia cells, were performed. Secondary response was stimulated by some but not all leukemic cells, suggesting that the PLT technique may provide a means for identifying the specificity of a system of leukemia-associated antigens distinct from the recognition of allogeneic histocompatibility specificities. However, the apparent specificity for subclasses of leukemic cells could also be explained by "self modification" (possibly by a viral antigen) with secondary phase restriction requiring both histocompatibility determinants and the blast or viral antigen for secondary stimulation.

Data analysis

Three new methods were advocated for PLT data analysis. Franks and Bradley (Franks & Bradley 1977) described a method based on the

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calculation of error estimates. First, Log_e transformation of the data was performed in order to eliminate the difference in variance associated with low and high secondary response CPM. The transformed data was then subjected to a three-way analysis of variance test. This gave a residual mean square value which was used in the calculation of a standard error of group means. A series of least significant differences were then calculated using a series of critical values obtained from Harter's tables (Harter 1960). Critical values for a given number of means at the appropriate degrees of freedom were then multiplied by the standard error of group means to give least significant differences. These least significant differences were then used to perform multiple range testing (Duncan 1955). Each range of restimulation responses with given responder could be objectively divided into clusters, the members of which were not significantly different at a given probability level.

A second method was proposed by Rubenstein (Rubenstein et al. 1977). This new method was a further development of Piazza's cluster analysis (Piazza & Galfre 1975) which in turn was based on Fisher's method for discriminating analysis (Fisher 1936). By this method, each group of values (beginning with a group of just 1 value) in a series of responses was compared with each other group of that series. In principle, the analysis was based on an analysis of variance by which the sum of squares value realized an optimum.

A third procedure was proposed by Sheehy (Sheehy 1978). Responses were clustered either into one group with low cpm (the majority) or into a second group with intermediate or high cpm. The low group appeared to be almost normally distributed. The working model proposed was that the low cpm group clearly did not share any of the LD priming determinants with the reference cell. Essentially the method was performed by grouping

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the lowest 1/3 of secondary responses and eliminating any high values identified by a test of kertosis. If none of the values fell outside the normal group, the next highest value was added and the kertosis value recomputed. This process of progressively adding the next highest value was continued until a value outside the major low cpm group was identified. All values which fell outside the low group were said to share at least some specificities with the initial priming (reference) cell.

There are a number of other methods currently in use. One such technique is primarily based on the double blind scoring of results using arbitrary division of results into clusters by two independent observers. Relatively high secondary stimulation is considered to demonstrate sharing of specificity between the reference cell (primary stimulator) and secondary stimulator and conversely low stimulation represents lack of specificity sharing. A second technique, introduced by Thomsen, is based on performing experiments using a number of primed cells and secondary stimulators in a matrix (Thomsen et al. 1976). Each value is normalized to the maximum response of each primed cell followed by normalization to the maximum for each stimulator and the normalized values are grouped as high or low-level responses.

Although each method appears promising, extensive testing of the statistical methods with PLT data are necessary. A clearer understanding of the genetics of the PLT response is likely necessary before any one method of analysis can become accepted.

II. Genetics

It is most difficult to develop a sound method of data analysis without a thorough understanding of the genetics of the system. It is

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an equally difficult problem defining the genetics of such a complex system without an unbiased method of defining positives and negatives. Nonetheless certain fundamental principles are evolving.

In general, it was suggested that the PLT discriminates at least three different genetic systems: (1) HLA-D region determinants perhaps recognizing the same determinant as defined by homozygous typing cells; (2) determinants within the MHC but likely separate from the HLA-D region; and (3) determinants segregating independently of the major histocompatibility complex (MHC). Many laboratories were able to demonstrate methods of priming that resulted in PLT typing producing very high correlations with HLA-D typing by homozygous typing cell methods.

A number of methods were shown to be useful for generation of these HLA-D associated primed cells. First, priming can be performed with lymphoid cells from family members where primary responder and stimulator share one major histocompatibility complex haplotype. To attain HLA-D associated specificity in family typing, PLT combinations are generated and a preliminary screen against a random panel is performed to identify those combinations where highly discriminant PLT cells are generated. About 1/3 of the PLT cells produced by intra-familial combination are highly discriminant cells (Sondel & Bach 1977). However, 2/3 of the primed combinations are discarded as they produce relatively high secondary responses (when challenged in secondary phase) to many individuals and do not give clearly separable (discriminant) high and low level responses. The reason for this lack of discrimination in two thirds of PLT cells generated by one haplotype priming may be due to either additional MHC loci, non MHC genes, or a complexity of the HLA-D gene products. The use of multiple combinations (PLT cells generated in a number of families) which identify a single cluster of individuals

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who share an HLA-D determinant is helpful in eliminating the non-HLA-D effects in PLT typing.

A number of other priming techniques, which take advantage of known HTC typing have been used successfully to generate PLT cells apparently specific for HLA-D determinants. Various combinations of homozygous typing cells (HTC) and HLA-D typed heterozygous cells have been used in stimulator-responder pairs attempting to generate cells primed against a single HLA-D difference (Hirschberg, et al. 1977, Thomsen et al. 1976, Reinsmoen et al. 1977, Jaramillo et al. 1977, Hartzman et al. 1977). Although many of these primary combinations produce useful cells, the most consistent correlations with HTC typing occurred when both responder and stimulator cells were HTC's. In fact, nearly all PLT's generated solely with HTC's are highly discriminant and specific for the appropriate HLA-D type. Of interest, the use of HTC's as responder cells and heterozygous cells sharing one -D specificity as stimulators produced PLT cells which were frequently as specific as those generated with the use of HTC's as both primary phase responder and stimulator. However, in some cases, the HTC-heterozygous combinations were restimulated by cells not bearing the appropriate HLA-D type, suggesting priming to more than one specificity. The success of gener- ating PLT responses which were highly correlated with HTC typing where HTC's were used in the priming phase suggests that these HTC's may well have gene restrictions in addition to HLA-D homozygosity which allows the successful use both in primary MLR typing and PLT. Thus, homozygous typing cells may be initially selected on the basis of both homozygosity at HLA-D and restriction on non-HLA-D stimulating specificities.

Wank presented a method of intra-HLA-D group priming, in which two HTC of the Dw4 group were sensitized to each other. By neutralizing

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the responses to the HLA-D locus, non-HLA-D determinants could be iden-
tified (Wank et al. 1978). Suciu Foca and Rubenstein presented PLT data
from families serotyped for HLA-A, B and C and typed for HLA-D by the
HTC method. Priming was performed either by stimulating lymphocytes
from one individual with a second, unrelated, individual who differed by
a single HLA-D specificity or intrafamilial priming. In these studies,
some individuals who clearly did not possess the appropriate HLA-D type
for secondary stimulation and did not share an HLA-D type with the
reference cell were able to induce a secondary response similar to that
induced by the reference cell. However, other family members who shared
this MHC haplotype did not cause secondary stimulation. Thus, it appeared
that at least one gene segregating independently of MHC is capable of
causing both cell priming and secondary response. These studies postulate
genetic loci independent of the MHC affecting the PLT, however, the
initial data could be explained by a great deal of complexity of a
single genetic region.

A number of studies were carried out to determine gene dose
effect in PLT. A cooperative study between the Madison and Copenhagen
groups where heterozygous cells were primed with homozygous cells demon-
strated greater secondary stimulation by homozygous cells than heterozy-
gous cells with the appropriate HLA-D specificity (Bach et al. 1976).
However, this gene dose effect was not confirmed by HTC-HTC or HTC-
heterozygote priming in studies at the Naval Medical Research Institute
or University of Tübingen (Jaramillo et al. 1977). In addition, family
studies by Bradley and by Suciu-Foca and Rubenstein did not demonstrate
gene dose effect in PLT.

B CELL ALLOANTIGENS

As the understanding of B-cell alloantigens was still preliminary,
generalizations of the relationship between these specificities and PLT

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could not be made. However, Sasportes and Thorsby were both able to demonstrate strong associations between specificities defined by PLT, HTC and B-cell typing, and Sasportes reported a closer association between PLT and B-cell serologic typing than HTC typing. Hirschberg and Thorsby had used sera which were highly associated with HLA-D typing by HTC and PLT methods and found them capable of specifically inhibiting the PLT secondary phase stimulation of challenge cells known to share HLA-D specificity with the reference cell (Hirschberg et al. 1977).

COMMENT

The genetic interpretation of third party restimulation is still hampered by technical and biological difficulties as well as a lack of uniform method of data analysis. Methods of priming large uniform batches of PLT cells must be developed. The intricacies of cell concentrations and timing of both primary and secondary phases must be better understood. Much is needed in the understanding of the fundamental cell-cell interactions leading to in vitro priming. An optimum method of data analysis must be developed, but the analysis may have to wait until there is a more thorough understanding of the basic genetics and cellular interactions. A great deal has been defined since the introduction of PLT in humans, yet a great deal more needs to be known before the enormous value of in vitro cell priming can be fully realized.

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IN VITRO CHARACTERIZATION OF THE PLT CELL

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The Primed Lymphocyte Test (PLT) depends on in vitro priming of one lympho-
cyte population against those unshared cell surface specificities of a second
lymphoid cell. Primed human cells can be generated against HLA restricted normal
cells, biologically altered (leukemic) cells, or chemically altered cells. As
these PLT cells are generated in a mixed lymphocyte culture (MLC), it was felt
that the PLT responder cell would have the characteristics of the MLC responder
cell. A specific anti-human T cell antibody used at a concentration which
totally removed all responder primary MLR ("T") cells had no effect on PLT cells,
and complement-dependent cytotoxicity using this reagent did not kill these PLT
cells. However, the PLT cells could absorb the antisera when an extremely high
cell-to-sera ratio was used, whereas relatively few peripheral T cells could
remove the activity. Thus, the in vitro primed cell may be derived from a sub-
population of cells, which differs from the MLR responder cells, may become
resistant to the treatment of antisera, or may develop an altered cell surface
receptor. The trinitrophenyl (TNP) group has been used to modify cell surface
components in order to elucidate the nature of lymphocyte-mediated reactions.
Preliminary experiments with TNP-modified human lymphocytes in MLC and PLT
indicate that this tool will provide additional information about the receptor(s)
involved in these reactions and their relationship to the other products of the
human histocompatibility complex. (ONR Contract Nos. N00014-76-C-1173 and
N00014-77-C-0747.) PUblished in Tissue Antigens, Vol. 10, p. 164, as part of
the summary of the 7th International Histocompatibility Testing Workshop.

HISTOCOMPATIBILITY TESTING USING HOMOZYGOUS TYPING CELLS

AND PRIMED LD TYPING

R.J. Hartzman, A. Ahmed, P. Romano, D.M. Strong, F. Pappas and K.W. Sell

Identification of HLA-D specificities of a random population with
the use of homozygous typing cells (HTC).

Lymphocytes from 125 individuals from a random population which had
been previously serologically typed for HLA-A, -B and -C were further char-
aracterized for their HLA-D specificities using HTC. Multiple experiments
were performed in large matrices using 30-50 responder cells and 30-60
stimulator cells. The stimulator cells consisted of HTC and serologically
typed HLA-A, -B homozygous and HLA-D unknown cells. Approximately 25%
of the HLA-A and -B homozygous cells were found to function as HLA-D homozygous
typing cells. Data from these multiple experiments were used to determine
the genetic association of HLA-D with various other surface antigens, as
outlined below.

Association of random homozygous HLA-A and -B cells with their HLA-D
specificity.

As seen in Table I, data analysis of the matrix experiments showed that
certain HLA-A, -B homozygous cells were associated with a specific HLA-D
type (e.g., HLA-A1, -B8 HTC and DW-3, or probably splits of DW-3, etc.).

TABLE I

Associations Found Between HLA-B and -D of HTC

HLA-B	HLA-D
BW-35 or B-12	DW-1
B-7	DW2
B-8	DW-3
B-12 or BW-15	DW-4
B-12	DW-5
B-12 or B-14 or BW-17	DW-107-17a
B-12	New
B-27	New
BW-40	New

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It is of interest that the HLA-A-29 specificity, when found to occur
with the HLA-B-12 specificity, formed a combination which was nearly always
associated with the DW-107-17a HLA-D complex. This association may be due
to a haplotype linkage dissociation, but could also represent an HLA-A, -B
and -D gene product interaction. HLA-D-108 represented a difficult speci-
ficity, as few members of the random panel were typed by these cells and
responder cells which gave typing responses to one of these cells did not as
a rule type with other HLA-D-108 typing cells. Further, it was determined
that certain HLA-A, -B homozygous cells did not type for any of the known
HLA-D specificity. These HTC comprised of those bearing the HLA-A-28, -B-12
haplotype, or those bearing the HLA-B-27; and, lastly, those bearing the
HLA-B-40 specificity. Presumably, these could be HTC of previously unde-
fined HLA-D specificities.

HLA-D type association and cross reactivity

Certain HLA-D specificities occurred together, such as HLA-DW-3 and -107,
as did HLA-DW-2 and -6. Further, with the HLA-DW-6 HTC used in these experi-
ments, there appeared to be frequent cross reactivity to cells bearing DW-5,
even when associated with another DW specificity, such as DW-3 or DW-107.
Likewise, PLT cells generated against either DW-5 or DW-6 frequently showed
cross reactivity against the converse specificity.

The PLT system

Primed LD typing was extensively studied. The basic technical parameters,
such as cell concentration media requirements and timing of primary and
secondary harvest, were established. The primary phase culture could be
successfully primed with as little as 3×10^6 irradiated stimulator cells
and a like number of responder cells in 3 ml of tissue culture media, or as
many as 400×10^6 stimulator and responder cells in roller bottles with 200 ml
of media. Many schedules of media exchange were attempted; however, the most

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consistent results were obtained by exchanging media only when indicated by acidic (orange-red color of phenol red) and never allowing the cultures to become too acidic (yellow). Primary phase cultures could be harvested on days 8-14, although the most consistent results were found by harvesting on the 10th day. Each primary phase culture is somewhat unique and a true optimum harvest can only be precisely timed by performing daily blast counts and harvesting the culture after the blast count drops to approximately 5%. However, within the 8-14 day initial harvest period, the specificity of the PLT cells does not seem to be altered. Secondary cell concentrations nad harvest intervals gave acceptable results over a relatively wide range. The number of secondary phase PLT cells could be varied from 12.5 x 10^3 cells in 0.2 ml media in microtiter plates to over 200 x 10^3 responder cells. Like-wise, the stimulator cells could be varied from 12.5 to 200 x 10^6. The secondary phase culture could be harvested from 24-84 hours after initiation. Unfortunately, there is a fairly wide variation of optimum secondary culture intervals, some cells peaking at 36 hours and others peaking at 72 hours. In general, the greatest selectivity could be obtained by use of the earliest harvest intervals and lowest cell concentrations; but, the use of low numbers of cells and short culture intervals resulted in greater culture-to-culture variability. The most consistent combination of cell concentration and har-vest interval was found to be 25 x 10^3 PLT cells plus 50 c 10^3 stimulator cells labeled at 48 hours and harvested at 60 hours.

The PLT cell was examined for several characteristics. The effect of irradiation on the PLT system was determined for both the PLT cell itself and the secondary phase challenge cell. Irradiation of secondary phase stimulators was found unnecessary. No consistent improvement in specificity of the secondary response was found in over 50 combinations. None of 32 unirradiated normal lymphocytes responded after 60 hours of incubation to

PLT cells treated with 5,000 rads (^{60}Co). PLT cells were found to be relatively more radioresistant than resting peripheral lymphocytes.

Both PLT and resting lymphocytes (MLR responder cells) were examined for their susceptibility to lysis with rabbit antihuman thymic lymphocyte antigen (HTLA). Anti-HTLA in concentrations high enough to be cytotoxic to all MLD responder T cells had no effect on primed cells. No increased specificity in the challenge phase occurred when PLT cells were treated. However, anti-HTLA could be partially absorbed by the use of extremely high PLT cell-to-serum ratios (300 x 10^6 PLT cells absorbed 35% of the anti-HTLA activity from 10 λ of anti-HTLA). Furthermore, low dose anti-HTLA can cause stimulation of a primary mixed leukocyte reaction and subsequent generation of a PLT cell. This anti-HTLA generated PLT cell has no secondary specificity. Thus, the PLT cell may possess a derivative of the MLR receptor; however, this receptor is altered (or the cell becomes resistant for other reasons) during the process of priming, producing a cell which is resistant to anti-HTLA.

Development of the hybrid HTC-PLT system for the generation of highly discriminant PLT cells.

Approximately one-third of the PLT cells generated by lymphocytes from a parent-child combination produced highly selective typing cells. This low yield of specific cells by intrafamilial priming led to the use of defined HLA-D cells as stimulator cells against unknown responder cells by several laboratories. This was further refined by the use of a hybrid HTC-PLT system whereby HTC were used as primary phase responder cells against either another HTC of a different HLA-D specificity or a heterozygous cell sharing one HLA-D specificity. Data generated using this system resulted in a very high frequency of discriminant PLT cells with restricted specificity and is considered as the method of choice.

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The use of HTC as responder cells and typed heterozygotes as stimulators allows the possibility of generating PLT cells specific for currently unde-fined specificities (e.g., HLA-D 1/1 α 1/?). About one-half of these PLT cells appear to be restimulated by cells with undefined HTC specificities, suggesting priming for a new specificity. However, proof of new specifici-ties will have to await family studies. The remainder of these PLT cells appear to type for currently known specificities and thus may represent either some dichotomy between PLT and HTC testing or a mistyped reference cell.

HLA-D and -B cell serology

Further evidence of the restriction of HTC specificity has been found by the B-cell typing of these cells. HTC, which show highly discriminant specificity, were killed only with B-cell sera which fell within the appro-priate B-cell HLA-D association groups. However, HTC, which typed only a percentage of heterozygotes with the appropriate specificity defined by previous HTC typing, demonstrated frequent extra B-cell reactions. In one case, these "extra" reactions were related to a second B-cell HLA-D associa-tion group; but in three other cases, the extra reactions were not related to any known HLA-D association group.

Thus, there appears to be a complex series of relation between HTC, PLT and B-cell typing. PLT appears to type for HLA-D, as defined by HTC (or an associated genetic locus), but may also type for non-HLA-D specifici-ties. Many B-cell sera have been shown to be closely correlated with HTC assigned HLA-D specificity; yet, as discussed earlier, the sera probably type for a genetic region which is closely linked, but not identical, to HLA-D. Extensive studies with serologic adsorptions and, in addition, typing of MHC recombinant familieis will be needed to further defined these complex associations.

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
The primary goal of this work was to study the human histocompatibility
system and to develop methods for large-scale accurate tissue-typing for
support of ongoing kidney and bone marrow transplantation programs within
the Navy. The research which we have initiated is directed toward the
problem of examining the HLA-D antigenic system, defining the HLA-D locus
antigens, and cataloging them in such a manner that individuals could be
easily typed. The following has been accomplished:

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1) establishment of reproducible routine typing capacity;
2) development of methods for identification and procurement of homozygous typing cells (HTC);
3) establishment of cryopreservation techniques for long-term storage of HTC; and
4) development of the hybrid HTC-PLT system for the rapid identification of HLA-D types.